

Bio-Plex Pro[™] RBM Kidney Toxicity Assays

Instruction Manual

For technical support, call your local Bio-Rad office, or in the U.S., call 1-800-424-6723.
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Introduction

Early and Accurate Detection of Kidney Toxicity

Acute kidney injury (AKI) is frequently caused by trauma to the kidney due to drug exposure, an accident, or blood loss from a medical procedure. In contrast, chronic kidney injury (CKI) results from long-term diseases such as diabetes, high blood pressure, or from an inherited syndrome. Early detection of AKI along with proper treatment regimens is critical in the prevention of further loss of kidney function. The Bio-Plex Pro™ RBM kidney toxicity assays, developed in partnership with Myriad RBM, comprise a highly relevant set of biomarkers, found in urine, for early detection and characterization of kidney toxicity or injury. Myriad RBM's close collaboration with the Predictive Safety Testing Consortium (PSTC) was instrumental in the selection of markers found in these panels. Traditional markers such as serum creatinine (SCr) and blood urea nitrogen (BUN) are detectable days or weeks after kidney damage has occurred. The Bio-Plex Pro RBM kidney toxicity assays provide a tool to detect biomarkers that may reveal damage within hours, allowing drugs to be efficiently tested in both preclinical and clinical research settings.

Bio-Plex Pro RBM Kidney Toxicity Assays

The Bio-Plex Pro RBM assays are built on magnetic beads to enable robust quantification of multiple proteins in human, canine, and rat urine samples. For researchers working with limited sample volume, the capacity to multiplex in a small sample volume provides an effective option over the traditional ELISA. The use of magnetic (MagPlex) beads allows researchers to automate wash steps on a Bio-Plex Pro (or similar) wash station. Magnetic separation offers greater convenience, productivity, and reproducibility compared to vacuum filtration.

For more information please visit www.bio-rad.com/bio-plex.

Principle

Technology

The Bio-Plex® multiplex system is built upon the three core elements of xMAP technology:

- Fluorescently dyed magnetic microspheres (also called beads), each with a distinct color code or spectral address to permit discrimination of individual tests within a multiplex suspension. This allows simultaneous detection of up to 500 different types of molecules in a single well of 96-well microplate on the Bio-Plex® 3D system, up to 100 different types of molecules on the Bio-Plex® 200 system, and up to 50 different types of molecules on the Bio-Plex® MAGPIX™ system
- On the Bio-Plex 200 and Bio-Plex 3D systems, a dedicated flow cytometer with two lasers and associated optics to measure the different molecules bound to the surface of the beads. In the Bio-Plex MAGPIX, the entire sample load volume is injected into a chamber where the beads are imaged using LED and CCD technology
- A high-speed digital signal processor that efficiently manages the fluorescence data

Assay Format

The Bio-Plex Pro™ RBM kidney toxicity assays are essentially immunoassays formatted on magnetic beads. The assay principle is similar to that of a sandwich ELISA (Figure 1). Capture antibodies directed against the desired biomarker are covalently coupled to the beads. Coupled beads react with the sample containing the biomarker of interest. After a series of washes to remove unbound protein, a biotinylated detection antibody is added to create a sandwich complex. The final detection complex is formed with the addition of streptavidin-phycoerythrin (SA-PE) conjugate. Phycoerythrin serves as a fluorescent indicator, or reporter.

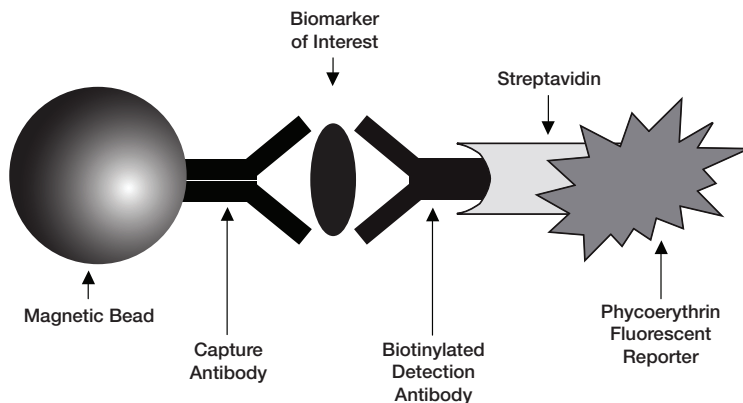


Fig. 1. Bio-Plex sandwich immunoassay.

Data Acquisition and Analysis

Data from the reactions are acquired using a Bio-Plex system or similar Luminex-based reader. When a multiplex assay suspension is drawn into the Bio-Plex 200 reader, for example, a red (635 nm) laser illuminates the fluorescent dyes within each bead to provide bead classification and thus assay identification. At the same time, a green (532 nm) laser excites PE to generate a reporter signal, which is detected by a photomultiplier tube (PMT). A high-speed digital processor manages data output, and Bio-Plex Manager™ software presents data as median fluorescence intensity (MFI) as well as concentration (pg/ml). The concentration of analyte bound to each bead is proportional to the MFI of the reporter signal.

Kit Contents and Storage

Reagents Supplied

The Bio-Plex Pro™ RBM kidney toxicity assays are available in a convenient kit format that includes assay, reagent, and diluent components in a single box (Table 1).

Table 1. Contents of 1 x 96-well kits.

Component	Quantity	Volume	Volume After Reconstitution or Dilution
Capture beads (1x)	1 tube	1.4 ml	
Detection antibodies	1 vial	Lyophilized	4.8 ml
Standards mix	1 vial	Lyophilized	150 µl
Control 1 (high)	1 vial	Lyophilized	100 µl
Control 2 (low)	1 vial	Lyophilized	100 µl
Blocking buffer	1 vial	Lyophilized	1.5 ml
Standard diluent	1 vial	Lyophilized	1.0 ml
Sample dilution buffer	1 bottle	100 ml	
Assay buffer (10x)	1 bottle	60 ml	600 ml
Streptavidin-PE (10x)	1 tube	250 µl	2.5 ml
Assay plate (96-well flat bottom)	1 plate		
Plate seals	1 pack of 3		
Assay quick guide	1 sheet		
Product data sheet	1 sheet		

Storage and Stability

Kit contents should be stored at 2–8°C and never frozen. Coupled magnetic beads and streptavidin-PE should be stored in the dark. All components are guaranteed for a minimum of six months from the date of purchase when stored as specified.

Table 2. Recommended materials.

Item	Ordering Information
Bio-Plex® 200 system or Luminex system with HTF	Bio-Rad catalog #171-000205
Bio-Plex validation kit Note: Run the validation kit monthly to ensure optimal performance of fluidics and optics systems	Bio-Rad catalog #171-203001
Bio-Plex calibration kit Note: Run the calibration kit daily to standardize fluorescence signal	Bio-Rad catalog #171-203060
Bio-Plex Pro wash station For use with magnetic bead-based assays only	Bio-Rad catalog #300-34376
Bio-Plex Pro II wash station For use with both polystyrene (nonmagnetic) and magnetic bead-based assays	Bio-Rad catalog #300-34377
Bio-Plex handheld magnetic washer For use with magnetic bead-based assays only	Bio-Rad catalog #170-20100
Bio-Plex Pro flat bottom plates (40 x 96-well) For magnetic separation on the Bio-Plex Pro wash station	Bio-Rad catalog #171-025001
Titertube® micro test tubes For preparing replicate standards, samples, and controls prior to loading the plate	Bio-Rad catalog #223-9390
Microtiter plate shaker IKA MTS 2/4 shaker for 2 or 4 microplates or Barnstead/Lab-Line Model 4625 plate shaker (or equivalent capable of 300–1,100 rpm)	IKA catalog #320-8000 WWR catalog #57019-600
Bio-Rad® Aurum™ vacuum manifold For vacuum filtration	Bio-Rad catalog #732-6470
BR-2000 vortexer	Bio-Rad catalog #166-0610
Reagent reservoirs, 25 ml (for capture beads and detection antibodies)	VistaLab catalog #3054-1002 or VistaLab catalog #3054-1004
Reagent reservoir, 50 ml (for reagents and buffers)	VistaLab catalog #3054-1006
Pall Life Science Acrodisc: 25 mm PF syringe filter (0.8/0.2 µm Supor membrane)	Pall Life Sciences catalog #4187
Filter plate, 1 x 96 with clear plastic lid and tray	Bio-Rad catalog #171-304502

Other: 15 ml polypropylene tubes for reagent dilutions, calibrated pipets, pipet tips, sterile distilled water, aluminum foil, absorbent paper towels, 1.5 or 2 ml microcentrifuge tubes, and standard flat bottom microplate (for calibrating vacuum manifold).

Assay Workflow

Reconstitute lyophilized reagents, dilute assay buffer to 1x, prepare standards and samples

Add 10 μ l blocking buffer to all wells

Add 30 μ l standards, blank, samples, and controls to appropriate wells

Add 10 μ l 1x capture beads per well.
Incubate at 850 ± 50 rpm for 1 hr at RT

Wash 3x with 100 μ l assay buffer (1x)

Add 40 μ l reconstituted detection antibodies.
Incubate at 850 ± 50 rpm for 1 hr at RT

Do not aspirate after incubation

Add 20 μ l 1x streptavidin-PE.
Incubate at 850 ± 50 rpm for 30 min at RT

Wash 3x with 100 μ l assay buffer (1x)

Resuspend beads in 100 μ l assay buffer (1x).
Shake at 850 ± 50 rpm for 30 sec

Read plate on Bio-Plex system

Important Considerations

Instruments and Software

The assays described in this manual are compatible with all currently available Luminex-based life science research instruments. Assays can be read and analyzed with either Bio-Plex Manager™ software or Luminex xPonent software.

Assay Procedures

Pay close attention to vortexing, shaking, and incubation times and to Bio-Plex® reader PMT (RP1) setting, as these have been optimized specifically for each assay panel.

Assay Quick Guide

Each assay kit comes complete with a printed Bio-Plex Pro RBM Kidney Toxicity Assays quick guide (bulletin #10028259), which can be used to set up and prepare a full 1 x 96-well assay plate. Users can also download a copy at www.bio-rad.com/bio-plex.

Bead Regions and Multiplexing Compatibility

- Bead regions for all analytes are listed in the Read Plate section
- Do not mix analytes between different kidney toxicity panels, or with other Bio-Plex assay panels or reagents

Detailed Instructions

The following pages provide detailed instructions for each step of the assay procedure, including preparation, running the assay, and reading the plate with Bio-Plex Manager™ and Luminex xPONENT software.

1. Plan Plate Layout

Determine the total number of wells in the experiment using the Plate Layout Template on page 28 or the Plate Formatting tab in Bio-Plex Manager™ software. A suggested plate layout is shown in Figure 2, with all conditions in duplicate.

1. Assign standards to columns 1 and 2, with the highest concentration in row A and the lowest concentration in row H.
2. Assign the blank to wells A3 and A4. The blank should consist of standard diluent alone and be processed in the same manner as sample and standard wells. Note that Bio-Plex Manager automatically subtracts the blank (B) MFI value from all other assay wells.
3. User-specific controls, as well as the quality controls supplied in the kits, are assigned to wells in columns 3 and 4.
4. The remainder of the plate is available for samples.

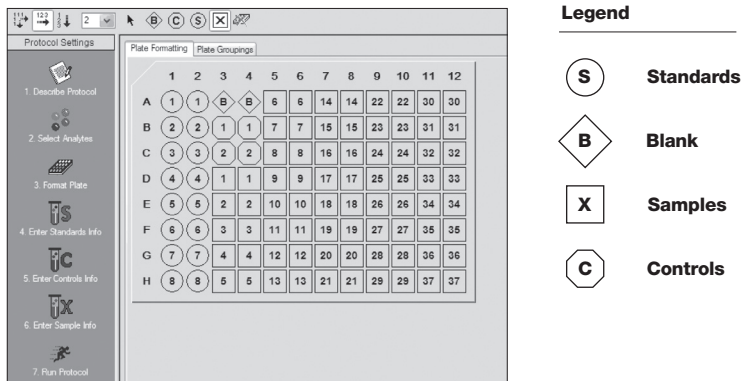


Fig. 2. Suggested plate layout. For detailed instructions on plate formatting in Bio-Plex Manager, see section Read Plate.

2. Prepare Instrument



These directions are specific for the Bio-Plex® 100/200 reader. To prepare either a Bio-Plex 3D or Bio-Plex® MAGPIX™ reader, consult their respective user manuals.

Start up and calibrate the Bio-Plex system with Bio-Plex Manager™ software prior to setting up the assay. The calibration kit should be run daily or before each use of the instrument to standardize the fluorescent signal. For instructions on using other xMAP system software packages, contact Bio-Rad Technical Support.


Note: While the instrument is warming up, bring the 10x assay buffer and sample dilution buffer to room temperature. Keep other items on ice until needed. Also, begin to thaw frozen samples.

The validation kit should be run monthly to ensure performance of fluidics and optics systems. Refer to either the software manual or online Help for directions on how to conduct validation (Bio-Plex 100, 200, or similar).


Start Up System (Bio-Plex 100, 200, or similar)

1. Empty the waste bottle and fill the sheath fluid bottle before starting if high throughput fluidics (HTF) are not present. This will prevent fluidic system backup and potential data loss.
2. Turn on the reader, XY platform, and HTF (if included). Allow the system to warm up for 30 min (if not already done).
3. Select Start up  and follow the instructions. If the system is idle for 4 hr without acquiring data, the lasers will automatically turn off. To reset the 4-hr countdown, select Warm up  and wait for the lasers/optics to reach operational temperature.

Calibrate System

1. Select Calibrate  and confirm that the default values for CAL1 and CAL2 are the same as the values printed on the bottle of Bio-Plex calibration beads. Use the Bio-Plex system low RP1 target value.

2. Select OK and follow the software prompts for step-by-step instructions for CAL1 and CAL2 calibration.

Note: In Bio-Plex Manager version 6.1 and higher, startup, warm up, and calibration can be performed together by selecting the “Start up and calibrate” icon. 

3. Prepare Wash Method

Bio-Plex Pro™ assays are compatible with both magnetic separation and vacuum filtration methods. However, for best results, we recommend performing the assays in a flat bottom plate with magnetic separation.

Table 3. Summary of compatible wash stations and plate types.

Wash Method	Wash Station	Assay Plate
Magnetic separation	Bio-Plex Pro Bio-Plex Pro II (use MAG programs) Bio-Plex® handheld magnetic washer	Flat bottom plate
Vacuum filtration	Bio-Plex Pro II (use VAC programs) Vacuum manifold (manual)	Filter plate

Setting up the Bio-Plex Pro or Bio-Plex Pro II Wash Station

The wash station does not require calibration; however, it should be primed before use. For more information, refer to the Bio-Plex Pro and Pro II wash station quick guide (bulletin #5826).

1. Install the appropriate plate carrier on the wash station.
2. Use the prime procedure to prime channel 1 with wash buffer.

Setting Up the Bio-Plex Handheld Magnetic Washer

Place an empty flat bottom plate on the magnetic washer by sliding it under the retaining clips. Push the clips inward to secure the plate. Make sure the plate is held securely. If needed, the clips can be adjusted for height and tension. For detailed instructions, refer to the user guide (bulletin #10023087).

Setting up a Vacuum Manifold

Calibrate the vacuum manifold by placing a standard 96-well flat bottom plate on the unit and adjusting the pressure to -1 to -3" Hg. In general, 100 μ l liquid should take 3-4 sec to clear the well. For more detailed instructions, refer to bulletin #10005042.

4. Prepare Reagents

1. Reconstitute the following lyophilized reagents in dH₂O before use according to the table below.

Table 4. Reagent volume.

Reagent	dH ₂ O Volume
Standards mix	150 μ l
Control 1	100 μ l
Control 2	100 μ l
Blocking buffer	1.5 ml
Standard diluent	1.0 ml
Detection antibodies	4.8 ml

- a. Allow vial to sit at room temperature for a minimum of **5 min**, not to exceed **30 min**.
 - b. Mix by **vortexing** at a medium setting.
2. Bring the 10x assay buffer to room temperature (RT).
 - a. Mix by inversion to ensure all salts are into solution.
 - b. Prepare 1x assay buffer — dilute **1 part** 10x assay buffer with **9 parts** of dH₂O.

Dilution of Standard (1:3 Serial Dilution)

This procedure provides sufficient volume to run duplicate standard dilution curves. Ensure that each new standard is mixed well by vortexing before proceeding to the next dilution. Change tips between each dilution.

Note: The product data sheet in each kit lists the most concentrated point on the standard curve (S1). Enter the values and units into Bio-Plex Manager™ software as instructed in the Read Plate section.

1. Label 8 polypropylene tubes **S1** through **S8**. Label an additional tube Blank.
2. Transfer the reconstituted standard into the tube labeled “**S1**.”
3. Add the appropriate amount of the standard diluent into the labeled tubes according to Figure 3 (this will be sufficient for duplicate standard curves and blanks).
4. Prepare working standards (**S2–S8**) by 1:3 (threefold) serial dilution. Transfer the appropriate volume of standard into each of the labeled tubes containing standard diluent.
5. **Vortex** each standard at a medium setting for **5 sec** before proceeding with the next serial dilution. Change pipet tip at each dilution step.

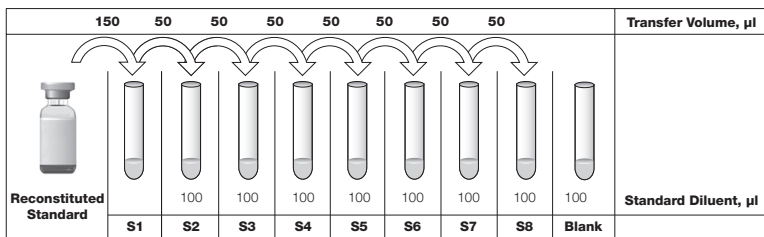


Fig. 3. Preparing a threefold dilution series with a single reconstituted standard.

5. Prepare Samples

General guidelines for preparing urine samples are provided here. For more information contact Bio-Rad Technical Support.

1. Centrifuge urine samples at 500 x g for 5 min to remove particulates.
2. Transfer the cleared samples into new aliquot tubes.
3. Place on ice for immediate use, or store as single-use aliquots at -70°C . Avoid multiple freeze-thaws.
4. Thaw frozen samples completely prior to dilution.
5. Within 1 hr after thawing, prepare sample dilutions in polypropylene tubes (such as Titertube[®] micro test tubes) as recommended in Table 5. Dilution scenarios provided are sufficient to run each sample in duplicate.
6. Equilibrate diluted samples to room temperature just prior to the start of the assay. Do not freeze diluted samples.

Table 5. Summary of recommended sample dilutions.

Panel	Sample Dilution	Volume of Urine Sample, μl	Volume of Sample Buffer, μl
Human Tox 1	1:4	20	60
Human Tox 2	1:50	10	490
Rat Tox 1	1:2	40	40
Rat Tox 2	1:50	10	490
Rat Albumin	1:10,000	5 (A. Prepare 1:100) 5 (B. Prepare 1:100)	495 495
Canine Tox 1	1:15	10	140
Canine Albumin	1:10,000	5 (C. Prepare 1:100) 5 (D. Prepare 1:100)	495 495

Note: Controls are ready to use after reconstitution. No dilution is needed.

6. Run the Assay

Considerations

- Bring all assay components and samples to room temperature before use
- Use calibrated pipets and pipet carefully, avoiding bubbles. Use new pipet tips for every volume transfer
- Pay close attention to vortexing, shaking, and incubation instructions. Deviation from the protocol may result in low assay signal and assay variability
- Assay incubations are carried out in the dark on a shaker at **850 ± 50 rpm**. Cover the plate with a plate seal and protect from light with aluminum foil

Table 6. Summary of wash options and protocols. After each assay step, select the appropriate Bio-Plex Pro™ wash station program or perform the appropriate manual wash step as summarized below.

Assay Step	Bio-Plex Pro or Pro II Wash Station	Bio-Plex Pro II Wash Station	Handheld Magnet or Vacuum Manifold
	Magnetic Program	Vacuum Program	Manual Wash Steps
Sample incubation			
SA-PE incubation	MAG x3	VAC x3	3 x 100 µl

Considerations When Using a Vacuum Manifold

- After each incubation, place the filter plate on a calibrated vacuum apparatus and remove the liquid by vacuum filtration
- To wash, add 100 µl wash buffer to each well and remove the liquid as before. Ensure that all wells are exposed to the vacuum
- Thoroughly blot the bottom of the filter plate with a clean paper towel between each vacuum step to prevent cross contamination
- Place the assay plate on the plastic plate holder/tray as needed
- Before each incubation, gently cover the plate with a new plate seal. Avoid pressing down over the wells to prevent leaking from the bottom

Assay Protocol: Dispensing of Reagents

1. Add **10 µl** of blocking buffer to all wells of the plate.
2. Add **30 µl** of the standard, blank, control, or sample to the appropriate well of the plate.
3. **Vortex** the capture beads at medium speed for **10–20 sec**. Add **10 µl** of the beads to all wells of the plate.
4. Cover plate with plate seal and protect from light with aluminum foil. Incubate on shaker at **850 ± 50 rpm** for **1 hr** at RT.
5. Wash the plate three times with **100 µl** 1x assay buffer.
6. **Vortex** the reconstituted detection antibodies at medium speed for **10–20 sec**. Add **40 µl** to each well.
7. Cover and incubate at **850 ± 50 rpm**, as in Step 4, for **1 hr** at RT.
8. Prepare the required dilution of streptavidin-PE (SA-PE) as outlined in Table 7.

Note: Volumes in the table are for an entire 96-well plate. Smaller volumes can be prepared, provided that dilution ratios are maintained.

Table 7. SA-PE dilution.

SA-PE Dilution	Volume of SA-PE, µl	Volume of 1x Assay Buffer, µl	Total Volume, µl
1:10	225	2,025	2,250

9. Add **20 µl** of diluted SA-PE to the required plate wells.
10. Cover and incubate at **850 ± 50 rpm**, as in Step 4, for **30 min** at RT.
11. Wash the plate three times with **100 µl** 1x assay buffer.
12. After the final wash, resuspend the beads in **100 µl** assay buffer. Cover plate as in Step 4 and shake the plate at **850 ± 50 rpm** for **30 sec**.
13. Remove the plate seal and read plate at low PMT (Bio-Plex® 200), standard PMT (Bio-Plex 3D), or default settings (Bio-Plex® MAGPIX™).

7. Read Plate


Bio-Plex Manager™ software is recommended for all Bio-Plex Pro™ assay data acquisition and analysis. Instructions for Luminex xPONENT software are also included. For instructions using other xMAP system software packages, contact Bio-Rad Technical Support or your regional Bio-Rad field applications specialist.

Prepare Protocol in Bio-Plex Manager Software Version 6.0 and Higher

The protocol should be prepared in advance so that the plate is read as soon as the experiment is complete.

A protocol file specifies the analytes used in the reading, the plate wells to be read, sample information, the values of standards and controls, and instrument settings. Protocols may be obtained from within Bio-Plex Manager software version 6.0 and higher or created from the File menu.

To create a new protocol, select **File**, then **New** from the main menu. Locate and follow the steps under **Protocol Settings**.

1. Click **Describe Protocol** and enter information about the assay (optional).
2. Click **Select Analytes** and create a new panel. Visually confirm the selected analytes and proceed to step 3.
 - a. Click the **Add Panel** button  in the Select Analytes toolbar. Enter a new panel name. Select **Bio-Plex Pro Assay Magnetic** from the assay dropdown menu. If using Bio-Plex Manager version 5.0 or lower, select **MagPlex** from the assay dropdown list.
 - b. Click the **Add** button. Enter the bead region number and name for the first analyte. Click **Add Continue** to repeat for each analyte in the assay.

For reference, bead regions for the individual assays are listed in Table 8.

- c. Click the **Add** button when the last analyte has been added and click **OK** to save the new panel.
- d. Highlight analytes from the Available list (left) and move to the Selected list (right) using the **Add** button. To move all analytes at once, simply click the **Add All** button.
- e. If some of the analytes need to be removed from the Selected list, highlight them and select **Remove**. If desired, it is possible to rename the panel by clicking on **Rename Panel** and entering a new panel name.

Table 8. Bead regions for Bio-Plex Pro RBM kidney toxicity panels.

Human Kidney Tox Panel 1	Bead Region	Human Kidney Tox Panel 2	Bead Region
Calbindin	64	Albumin	30
Clusterin	12	B2M	22
GST-p	25	Cystatin C	51
IL-18	21	NGAL	46
KIM-1	44	Osteopontin	20
MCP-1	15	TFF3	61

Canine Kidney Tox Panel 1	Bead Region	Canine Albumin Kit	Bead Region
Clusterin	12	Albumin	30
KIM-1	53		
MCP-1	15		
NGAL	46		

Rat Kidney Tox Panel 1	Bead Region	Rat Kidney Tox Panel 2	Bead Region	Rat Albumin Kit	Bead Region
Clusterin	12	B2M	22	Albumin	30
IL-18	21	Calbindin	62		
KIM-1	20	Cystatin C	44		
MCP-1	15	NGAL	46		
Osteopontin	52				

3. Click **Format Plate** and format the plate according to the plate layout created in Section 1 (Plan Plate Layout). To modify the plate layout, follow the steps below (see Figure 4).
 - a. Select the **Plate Formatting** tab.
 - b. Select the standards icon (S) and drag the cursor over all the wells that contain standards. Repeat this process for blanks (B), controls (C), and samples (X).

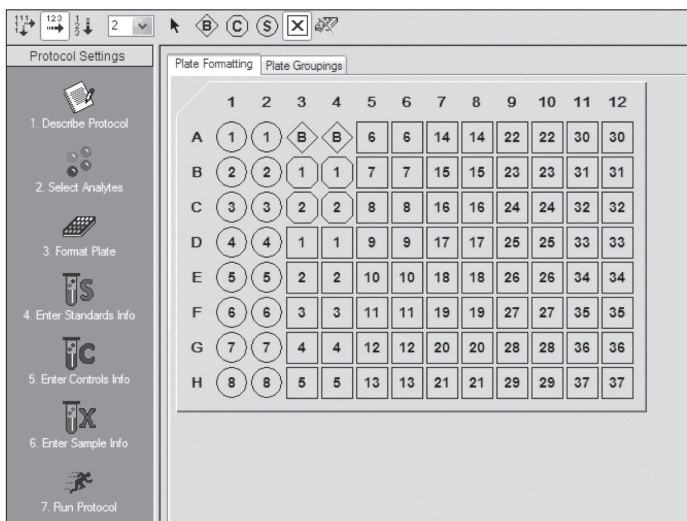


Fig. 4. Plate formatting.

4. Click **Enter Standards Info** in the Protocol Settings bar.
 - a. Enter the highest concentration of each analyte in the top row (labeled S1) of the table. S1 concentration information is listed in the product data sheet.
 - b. Enter a dilution factor of 3 and click **Calculate**. The concentrations for each standard point will be populated for all analytes in the table.

- c. Optional: enter the lot number of the vial of standards into the **Standard Lot** box and click **Save**.
5. Click **Enter Controls Info**.
 - a. For user-specified controls, select an analyte from the dropdown menu, then enter a description and concentration. Repeat for each additional analyte in the assay.
 - b. For the kit controls supplied, format the appropriate wells as controls and enter descriptions, but leave the concentrations blank. Alternatively, the controls can be formatted as samples with clear descriptions such as “quality control.” In any case, the expected control ranges provided on the product data sheet are not entered into Bio-Plex Manager software version 6.1 and earlier.
 6. Click **Enter Sample Info** and enter sample information and the appropriate dilution factor.
 7. Click **Run Protocol** and confirm that the settings follow Table 9.

Table 9. Read the plate using the appropriate instrument settings.



Instrument	RP1 (PMT)	DD Gates	Bead Events
Bio-Plex 100, 200*	Low	5,000 (low), 25,000 (high)	50
Bio-Plex 3D*	Standard	Select MagPlex beads	50
Bio-Plex® MAGPIX™	N/A, use default instrument settings		

* A similar Luminex-based system may be used.

- a. Confirm that data acquisition is set to **50 beads per region**.
- b. In Bio-Plex Manager Software prior to 6.1, go to Advanced Settings, confirm that the bead map is set to **100 region**, the sample size is set to **50 µl**, and the DD gates are set to **5,000 (Low)** and **25,000 (High)**. In Bio-Plex Manager software versions 4.0, 4.1, 4.1.1, and 5.0, check **Override Gates** and set the DD gate values as indicated.

Select **Start**, name and save the .rbx file, and begin data acquisition. The Run Protocol pop-up screen will appear. Click **Eject/Retract** to eject the plate carrier.

Acquire Data

1. Shake the assay plate at **850 ± 50 rpm** for **30 sec**, and visually inspect the plate to ensure that the assay wells are filled with buffer. Slowly remove the plate seal and any plate cover before placing the plate on the plate carrier.
2. Click **Run Protocol** and on the pop-up screen, select **Load Plate** and click **OK** to start acquiring data.
3. Use the **Wash Between Plates**  command after every plate run to reduce the possibility of clogging the instrument.
4. If acquiring data from more than one plate, empty the waste bottle and refill the sheath bottle after each plate (if HTF are not present). Select **Wash Between Plates** and follow the instructions. Then repeat the Prepare Protocol and Acquire Data instructions.
5. When data acquisition is complete, select **Shut Down**  and follow the instructions.

Data Analysis

Quality Controls

If the quality controls were run in the assay plate, open the results (.rbx) file, click on **Report Table**, and locate the control wells. Compare the observed concentrations against the lot-specific control ranges in the product data sheet.

Note: Expected control ranges are provided for reference and should be used as general guidelines. Actual results may vary for some operators. If the controls do not fall within the expected ranges, please refer to the troubleshooting section for possible causes and solutions.

Removing Outliers

Outliers are identified as standard data points that do not meet accuracy or precision requirements and should be considered invalid when performing curve fitting. As such, they should be removed to generate a more realistic and accurate standard curve. This may result in an extended assay working range and allow quantitation of samples that might otherwise be considered out of range (OOR).

In Bio-Plex Manager software version 6.0 and higher, outliers can be automatically removed by selecting the **Optimize** button in the Standard Curve window. In Bio-Plex Manager software 6.0 and earlier versions, outliers can also be manually selected in the Report Table. Visit online Help to learn more about the standard curve optimizer feature and how outliers are determined.

Previous Versions of Bio-Plex Manager Software

For instructions on using previous versions of Bio-Plex Manager software, please contact Bio-Rad Technical Support.

Luminex xPONENT Software

Luminex xPONENT software may be used to analyze Bio-Plex assays. Although guidelines are provided here, consult the xPONENT software manual for more details. Perform a system initialization with Luminex's calibration and performance verification kit, as directed by Luminex. Select **Batches** to set up the protocol and follow the information under Settings.

Note: The instrument settings described below apply to Luminex 100/200 and FLEXMAP 3D or Bio-Plex 3D instruments. For the Bio-Plex MAGPIX reader, use the default instrument settings.

1. Select MagPlex as the bead type for magnetic beads, which automatically sets the DD gates.
2. Volume = 50 μ l
3. Low PMT (Standard PMT).
4. Plate name: 96-well plate.
5. Analysis type: Quantitative; 5PL Curve Fit.
6. Number of standards: 8.

Select **Analytes** to set up the panel.

1. Enter "ng/ml" in the Units field.
2. Enter 50 in the Count field.
3. Select the bead region and enter the analyte name.
4. Click **Apply all** for Units and Count.

Select **Stds and Ctrl.**

1. Enter standard concentrations, lot number, dilution factor, and other information, as applicable.

After the assay is complete, select **Results**, then select **Saved Batches**.

Troubleshooting Guide

This troubleshooting guide addresses problems that may be encountered with Bio-Plex Pro™ RBM assays. If you experience any of the problems listed below, review the possible causes and solutions provided. Poor assay performance may also be due to the Bio-Plex® suspension array reader. To eliminate this possibility, use the validation kit to assist in determining if the array reader is functioning properly.

Possible Causes

High Inter-Assay CV

Standards were not reconstituted consistently between assays

Reconstituted standards and diluted samples were not stored properly

Bottom of filter plate not dry

Possible Solutions

Incubate the reconstituted standards for 30 min on ice. Always be consistent with the incubation time and temperature.

Reconstituted standards and diluted samples should be prepared on ice as instructed. Prior to plating, the reconstituted standards and diluted samples should be equilibrated to room temperature.

Dry the bottom of the filter plate with absorbent paper towel (preferably lint-free) to prevent cross-well contamination.

Possible Causes

High Intra-Assay CV

Improper pipetting technique

Reagents and assay components not equilibrated to room temperature prior to pipetting

Contamination with buffer during wash steps

Slow pipetting of samples and reagents across the plate

Low Bead Count

Beads clumped in multiplex bead stock tube

Possible Solutions

Pipet carefully when adding standards, samples, detection antibodies, and streptavidin-PE, especially when using a multichannel pipet. Use a calibrated pipet. Change pipet tip after every volume transfer.

All reagents and assay components should be equilibrated to room temperature prior to pipetting.

During the wash steps, be careful not to splash buffer from one well to another. Be sure that the wells are filtered completely and that no residual volume remains. Ensure that the microplate shaker setting is not too high. Reduce the microplate shaker speed to minimize splashing.

Sample pipetting across the entire plate should take less than 4 min. Reagent pipetting across the entire plate should take less than 1 min.

Vortex for 30 sec at medium speed before aliquoting beads.

Possible Causes

Low Bead Count

Vacuum on for too long when aspirating buffer from wells

Reader is clogged

Low Signal or Poor Sensitivity

Standards reconstituted incorrectly

Detection antibody or streptavidin-PE diluted incorrectly

High Background Signal

Incorrect buffer was used (for example, assay buffer used to dilute standards)

Accidentally spiked blank wells

Detection antibodies or streptavidin-PE incubated too long

Possible Solutions

Do not apply vacuum to the filter plate for longer than 10 sec after the buffer is completely drained from each well.

Refer to the troubleshooting guide in the Bio-Plex system hardware instruction manual (bulletin #10005042).

Follow the standard preparation instructions carefully.

Check your calculations and be careful to add the correct volumes.

Use standard diluent to dilute standards.

Do not add any antigens to the blank wells.

Follow the procedure incubation time precisely.

Possible Causes

Poor Recovery

Expired Bio-Plex reagents were used

Incorrect amounts of components were added

Microplate shaker set to an incorrect speed

High end saturation of the standard curve

Controls do not fall within expected ranges

Improper pipetting technique

Possible Solutions

Check that reagents have not expired. Use new or nonexpired components.

Check your calculations and be careful to add the correct volumes.

Check the microplate shaker speed and use the recommended setting. Setting the speed too high may cause splashing and contamination. Use the recommended plate shaker.

Make sure that correct shaker speed and incubation times are used. Remove S1 for data analysis if needed.

Make sure that the vial of controls is reconstituted at the same time as standards and in the correct diluent. Incubate for times indicated.

Pipet carefully when adding standards, samples, detection antibodies, and streptavidin-PE, especially when using a multichannel pipet. Use a calibrated pipet. Change pipet tip after every volume transfer.

Possible Causes

Impact of Sample Matrix

Negative MFI values in samples

Possible Solutions

If samples contain little or no analyte, negative values observed may be due to statistical variation. If assay drift is suspected, retest the samples by positioning them next to the standards. If contamination of standards is suspected, check the standard replicate value and be careful when adding samples to the wells. Matrix effects could also produce negative sample values.

Bio-Plex Manager™ software automatically subtracts the blank (B) FI value from all other assay wells. While this has no impact on observed concentrations of samples within the assay working range, it may result in a negative FI value if the blank's FI value is greater than either the standard or the sample value. If this is undesirable, then reformat the blank wells as sample (X) or control (C) in the protocol or results file.

Poor precision in serum and plasma sample measurements

Check if any interfering components such as heparin-based anticoagulant, additives, or gel from separators were introduced into the samples. Avoid using hemolyzed and heavily lipemic samples. Remove visible particulate in samples by centrifugation. Avoid multiple freeze-thaw cycles of samples.

Plate Layout Template

12								
11								
10								
9								
8								
7								
6								
5								
4								
3								
2								
1								
	A	B	C	D	E	F	G	H

Safety Considerations

Eye protection and gloves are recommended when using these products. Consult the MSDS for additional information. The Bio-Plex Pro™ assays contain components of animal origin. This material should be handled as if capable of transmitting infectious agents. Use universal precautions. These components should be handled at Biosafety Level 2 containment (U.S. government publication: Biosafety in Microbiological and Biomedical Laboratories (CDC, 1999)).

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The Bio-Plex suspension array system includes fluorescently labeled microspheres and instrumentation licensed to Bio-Rad Laboratories, Inc. by the Luminex Corporation.

Ordering Information

Detailed ordering information can be found at www.bio-rad.com/bio-plex.

Catalog #	Premixed All-In-One Multiplex Kit
Includes premixed magnetic capture beads, premixed detection antibodies, standards mix, 2-level controls, blocking buffer, standard diluent, sample dilution buffer, 10x assay buffer, 10x streptavidin-PE, 96-well flat bottom plate, plate seals, and instructions.	
171-ATR1CK	Bio-Plex Pro RBM Human Kidney Toxicity Panel 1 , 1 x 96
171-ATR2CK	Bio-Plex Pro RBM Human Kidney Toxicity Panel 2 , 1 x 96
171-KTR1CK	Bio-Plex Pro RBM Rat Kidney Toxicity Panel 1 , 1 x 96
171-KTR2CK	Bio-Plex Pro RBM Rat Kidney Toxicity Panel 2 , 1 x 96
171-KTR3CK	Bio-Plex Pro RBM Rat Kidney Toxicity Albumin Kit , 1 x 96
171-QTR1CK	Bio-Plex Pro RBM Canine Kidney Toxicity Panel 1 , 1 x 96
171-QTR2CK	Bio-Plex Pro RBM Canine Kidney Toxicity Albumin Kit , 1 x 96
Catalog #	Wash Stations and Accessories
300-34376	Bio-Plex Pro Wash Station , includes magnetic plate carrier, waste bottle, 2 buffer bottles
300-34377	Bio-Plex Pro II Wash Station , includes magnetic plate carrier, vacuum manifold plate carrier, waste bottle, 2 buffer bottles
171-020100	Bio-Plex Handheld Magnetic Washer , includes magnetic washer and adjustment hex tools for use in manual wash steps for all Bio-Plex magnetic assays
171-025001	Bio-Plex Pro Flat Bottom Plates , 40 x 96-well plates
171-304500	Bio-Plex Wash Buffer , 1.5 L
171-304502	Filter plate , pkg of 1, 96-well plate with clear plastic lid and tray, for Bio-Plex assays using the vacuum wash method, sealing tape not included
Catalog #	Software
171-001510	Bio-Plex Data Pro with Bio-Plex Manager Software (5 seats) , for multi-experiment analysis and advanced data visualization
171-001513	Bio-Plex Data Pro Software (5 seats) , for multi-experiment analysis and advanced data visualization
171-001523	Bio-Plex Data Pro Plus Software , contains all the features of Bio-Plex Data Pro software with added visualization, sharing, and analysis functionality
171-STND01	Bio-Plex Manager Software (1 seat) , for instrument data evaluation and optimization

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Finland 09 804 22 00 **France** 01 47 95 69 65 **Germany** 089 31 884 0
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